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14. ABSTRACT
In this project, we proposed to use tumor-specific T cells to produce an immunotoxin (IT) targeting tumor cells only when these T cells are specifically activated by the tumor. We use lentiviral vectors to modify tumor specific T cells with our immunotoxin. PEA based immunotoxins affect cell viability by ADP ribozilation of their elongation factor-2. To produce high titer of vector encoding the IT we generated a producer cell line resistant to PEA toxin. We have established stable cell lines of PEA-resistant producer cells. Using these stable cell lines, we have produced a high titer of IT-lentivirus preparation and transduced T cells with these vectors encoding the immunotoxin. We have characterized the transduced T cells to ensure that their phenotype and function was not impaired by the genetic modification and compared them to parental T cells. Finally, we have verified that transduced T cells produced the therapeutic immunotoxin that specifically kills our targeted tumor cells.

15. SUBJECT TERMS

Immunotoxin, tumor specific T cell, T cell activation, CD40L, antigen specific, Elongation factor

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INTRODUCTION.

Researchers have succeeded to enhance the effector function of monoclonal antibodies by coupling toxic moieties to the targeting portion of the antibody. These "warheads" have included radionucleotides and toxins (immunotoxins: IT). The antibody binds to the cell surface and the toxin is internalized into the cytosol, where it inhibits critical cell functions or damages the cell membrane, leading to cell death. The commonest IT are composed of the variable domain of a monoclonal antibody single (scFv) or double chain (dcFv), conjugated or fused via a linker sequence to a toxin that has been modified to decrease nonspecific binding to non-targeted normal cells. Although this approach has shown promise in animal models, toxicity issues have limited its clinical application. Only a small proportion of the drug reaches target cells after systemic injection, meaning that relatively high doses of IT are required to induce a significant biologic effect. Consequently, the increase in killing of target cells mediated by toxin is partly offset by increased toxicity. Non-specific clearance by liver and kidneys in particular may produce substantial and even fatal damage to these organs at doses optimal for anti-tumor activity. In hematological malignancies, for example, systemic injection of IT may cause vascular leak syndrome, thrombocytopenia, and liver damage. Here, we propose to minimize this toxicity by using tumor-antigen specific T cells to further target delivery of an immunotoxin, the *CD22-Pseudomonas exotoxin A* (CD22-PEA), which has already been used in a clinical setting. The toxin portion contains the translocating and ADP-ribosylating domains of PEA, and the native cell-binding portion is replaced with a CD22 scFv that directs targeting to B lymphocytes. CD22-PEA was tested in a Phase I trial in B-cell malignancies, but tumor responses, particularly in hairy cell leukemia, were offset by an unfavorable toxicity profile. The current project will use the anti-tumor activity of CD22-PEA while minimizing its adverse effects by delivery from T cells. Because the T cells we use are specific for tumoral antigen, they can only be activated when they encounter the tumor. By controlling the IT production with a promoter dependent on T cell activation, and using tumor specific T cells, we can limit the production and delivery of IT to the tumor site. This approach should increase the quantity of IT delivered to the tumor while preventing toxicity to the normal tissue.

BODY.

We have generated very encouraging data for the second task of this project. Using 293T that express mutated elongation factor, we have produce viral vectors encoding our Immunotoxin to efficiently transduce T cell lines. We have shown that such T cells are efficiently transduced and keep their native phenotype and function. We have reported our recent findings below in the order described in the original S.O.W

Aim 1: To generate cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigens LMP1 and LMP2, and to engineer these lymphocytes to produce an anti-CD22-toxin following T cell activation (using CD40L promoter).

Second Year:

Transduction level. T cells were activated with CD3 and CD28 antibodies then transduced with our lentivirus vectors. Transduction of the activated T cells (named OKT3) was confirmed using real time PCR. Primers were designed to amplify a region of the PEA immunotoxin gene. DNA was extracted from T cells transduced with LN-CMV-HA22, and the copy number per reaction of the integrated lentiviral vectors and the housekeeping albumin gene was assessed using the standard curve method. From this, the transduction level was calculated at 4.79×10^{6} vector copies/million cells.

Spontaneous lymphoproliferation. To confirm that the transduced cells had not lost their dependence on IL2, and thus were not able to spontaneously proliferate (become a tumor), unmodified T cells and T cells transduced with LN-CMV-HA22 were cultured in RPMI with 10% FBS but no IL2. Cell number and viability were assessed using trypan blue exclusion. Results are shown in Figure 1. By 10 days after the withdrawal of IL2, all cells in both the control non-transduced T cells and LN-CMV-HA22 transduced T cells cultures were dead. This confirms that transduction with the IT vector does not confer lymphoproliferative ability to the T cells, and adds to data suggesting gene modified cells are safe for patient use.

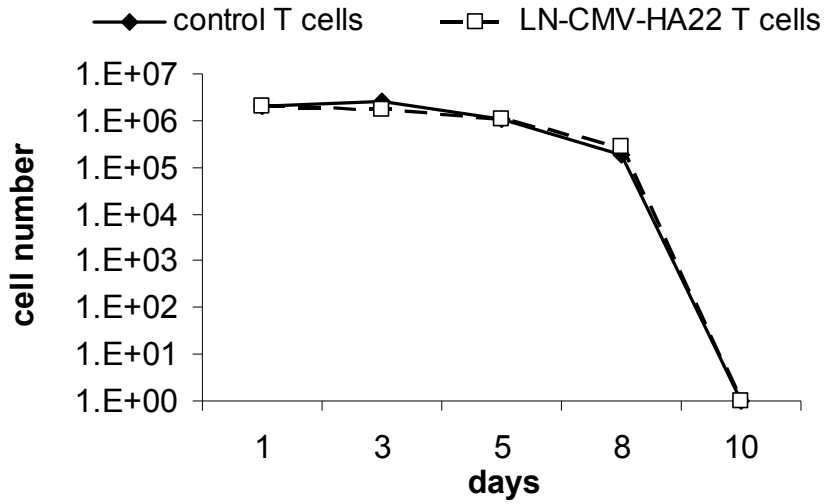

Figure 1: Lymphoproliferation in medium without IL2. Withdrawal of IL2 from the culture medium results in T cell death, regardless of transduction with an IT vector.

Cell phenotype. To confirm that transduction had not altered the phenotype of transduced T cells, the expression of cell lineage markers was assessed. Unmodified and LN-CMV-HA22 transduced T cells were stained with CD3, CD4, CD8, CD19 and CD56 antibodies and analyzed by flow cytometry. Results are shown in Table 1. As expected, the majority of the culture was CD3 positive T cells, with a greater number of CD8 than CD4 T cells. CD19 positive B cell numbers in the cultures were negligible, and there were comparable numbers of CD56 positive NK cells.

Table 1: Expression of cell surface markers on unmodified and transduced T cells.

Surface markers	Control T cells	LN-CMV-HA22 T cells
CD3	81.8	85.5
CD4	23.1	24.1
CD8	37.3	37
CD19	0.2	0.4
CD56	24.1	19.9

Cytokine production. We investigated the production of cytokines following cell activation, and compared activated T cells with activated IT transduced T cells. Antibodies specific for Interleukine-2 (IL-2) and Interferon gamma (IFN-γ) were used to assess our control-parental and transduced T cells. The cells were activated with the phytohemagglutin lectinn (PHA), and subsequently incubated with Brefeldin A for 16 hours to prevent protein export. Cells were fixed in paraformaldehyde, then intracellular staining performed for IL-2 and INF-γ. Cells were analyzed by flow cytometery for expression of the cytokines. Results are reported as a percentage of control T cells, and showed that transduced activated T cells have similar production of cytokine to control parental T cells, revealing that the transduction and synthesis of IT did not impair the production of the cytokines tested.

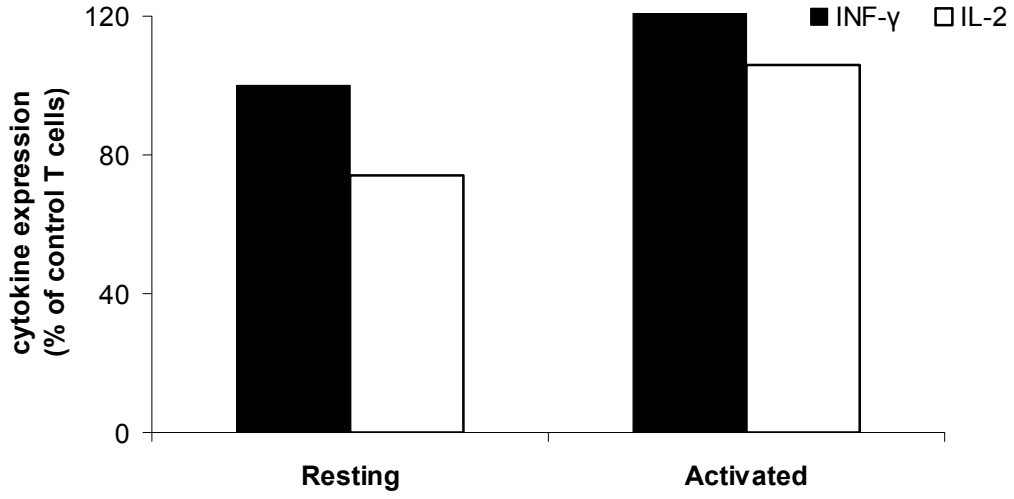

Figure 2: Expression of cytokines in resting and PHA activated T cells transduced with LN-CMV-HA22.

Apoptosis assay. To confirm that transduction with the IT vector did not affect the T cell capability for cell death following activation, an annexinV apoptosis assay was performed. Cells were activated overnight with 3µg /ml PHA, stained for annexin V and propidium iodide, and analyzed by flow cytometry. Results are shown in Figure 2. Transduced cells became apoptotic

(lower right quadrant) and died (upper right quadrant) following activation with PHA at the same rate as unmodified cells. This corroborates the data from the lymphoproliferation assay (Figure 1), showing that transduction with the IT vector does not confer a survival advantage to the modified cells, and thus supports the safety of this approach.

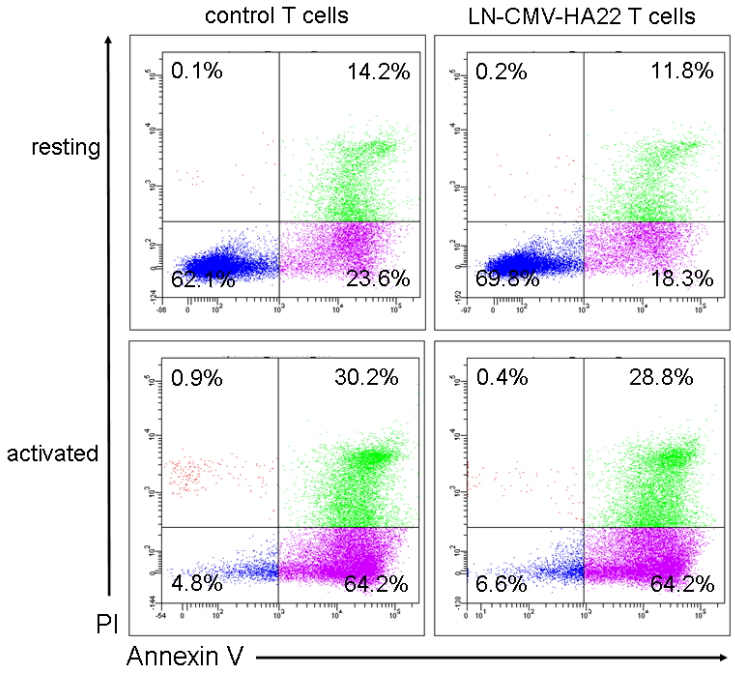

Figure 3: Apoptosis of unmodified and transduced cells following activation with PHA. Transduction with an IT vector did not affect the susceptibility of T cells to become apoptotic (lower right quadrant - purple) or die (upper right quadrant - green) following activation with PHA.

Chemokine receptor expression. To ensure that transduced cells would retain their ability to respond to chemokines, cells were assessed for chemokine receptor expression. Resting (IL-2 20U/ml) and activated (PHA 3µg/ml + IL-2 20U/ml) unmodified and LN-CMV-HA22 transduced T cells were stained with CCR7, CXCR4, CD25, CD31, CD62L and analyzed by flow cytometry. Results are shown in Table 2. Chemokine receptor expression was closely matched on transduced and unmodified cells, and was increased in both populations following activation. This demonstrates that transduction with the IT vector has not affected the ability of the T cells to receive chemokine signaling, and thus their potential to respond to immunological stimuli.

Table 2: Expression of chemokine receptors on unmodified and transduced T cells.

	control T cells		LN-CMV-HA22 T cells	
	resting	activated	resting	activated
CCR7	2.1	9.7	2.9	9
CXCR4	24.8	38	28.4	38.4
CD25	59.8	90.5	64	91.1
CD31	58.3	67.6	62.4	69.6
CD62L	86.9	65.3	76.9	52

Migration in response to chemokines. The transduction did not affect the expression of chemokine receptors, as detailed above. However, to ensure that the IT transduced T cells were capable of responding to those chemokines, a migration assay was performed. The assays were performed in transwell chambers with a 3µm pore polycarbonate membrane. Fresh or conditioned media was added to the lower chamber of the transwells, the membranes were placed on top, and $5x10^5$ unmodified or LN-CMV-HA22 transduced T cells in fresh media were added to the upper chamber. Conditioned media was collected from human macrophages which had been cultured overnight in 50ng/ml LPS, as activated macrophages secrete the chemokine SDF-1, the ligand for CXCR4. The cells were allowed to migrate for 4.5 h at 37°C in 5% CO_2, when migrated cells were collected and counted using trypan blue. Results are shown in Figure 3. Less than 15% of cells in control wells with fresh media only were able to cross the membrane during the culture period, while over 35% of cells transduced with the IT vector did so. Thus, the transduced cells retained their ability to respond to chemokine signaling, and migrated in equivalent numbers to unmodified cells.

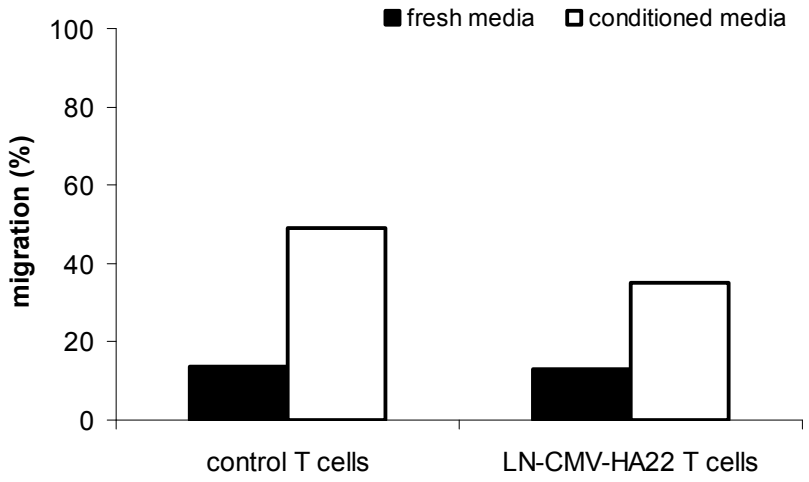

Figure 4: Migration of T cells in response to chemokines. T cells transduced with the IT vector retained their ability to migrate across membranes in response to conditioned media,

containing chemokines produced by activated macrophages. Migration in controls was less than 15%.

Proliferation. To ensure that the T cells were still capable of proliferation following transduction, proliferative capability following activation was assessed. Unmodified or LN-CMV-HA22 transduced T cells were cultured with 20U IL2/ml (resting), 50U/ml IL2, or CD3/CD28+20U IL2/ml (plate coated with 0.5µg/ml each Ab for 2 hrs) for 3 days at 37°C in 5% CO_2. The level of proliferation was then assessed using an ATP assay to measure metabolically active cells (Promega #G7570). IL2 at 50U/ml and stimulation with CD3/CD28 antibodies increased cell proliferation in cells transduced with the IT vector, as shown in Figure 4. This confirms that transduced cells retain their ability to proliferate in response to (antigen) stimulation, making them more effective therapeutically. However, the proliferative response was equivalent to that of unmodified cells, again supporting the data showing that transduction with the IT vector is a safe approach.

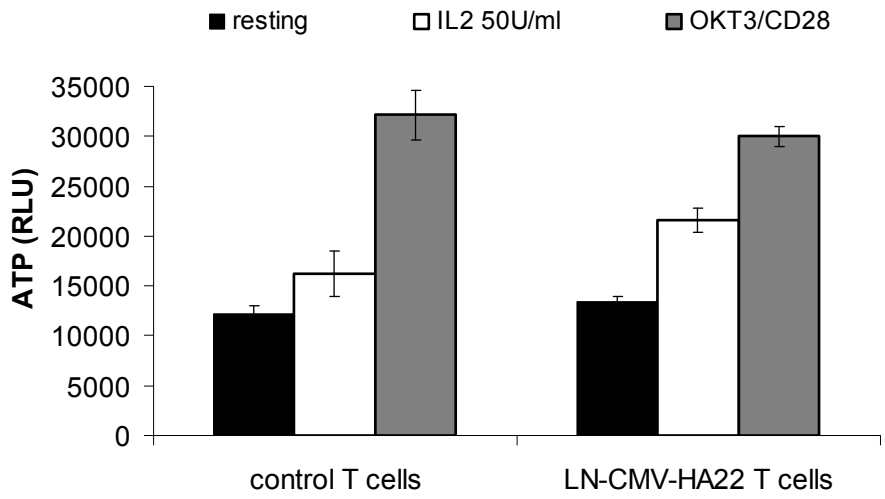

Figure 5: Proliferation of unmodified and transduced cells following stimulation with IL2 or CD3/CD28. T cells transduced with the IT vector retain their ability to proliferate in response to stimulation with 50U/ml IL2 or the T cells and CD28 antibodies. Data is expressed in relative light units.

Cytotoxicity. To confirm the lytic capability of the IT produced by T cells ~~cells~~, a supernatant cytotoxicity assay was performed. CD22 positive LCL and CD22 negative K562 cells were cultured for seven days at 37°C in 5% CO_2 with supernatant from unmodified or LN-CMV-HA22 transduced T cells. Half the culture volume was replaced with the appropriate fresh supernatant on days two and five. Cell number was assessed at seven days using trypan blue. Results are shown in Figure 5. The total cell number of the LCL culture was reduced by over 35%, compared to 5% in the K562 culture, showing the efficacy and specificity of the IT. As the LN-CMV-HA22 T cells from which the supernatant was harvested were transduced only once, and not enriched for the transgene in any way, efficacy will likely improve if these techniques are employed.

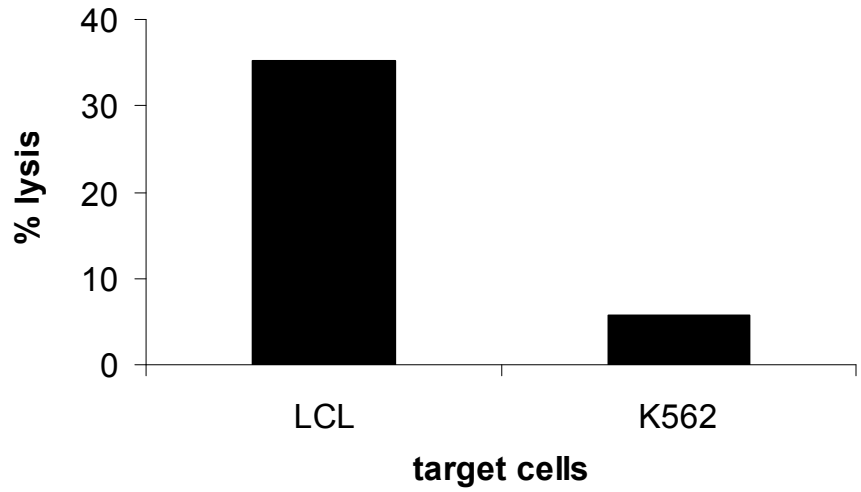

Figure 6: Specific cytotoxicity of th e immunotoxin against a CD22 positive lymphoproliferative cell line (LCL). Supernatant from T cells-LN-CMV-HA22 transduced T cells was cytotoxic against a CD22 positive LCL but not the CD22 negative K562 cell line. Lysis is displayed as a percentage of cells cultured in unmodified T cells supernatant.

KEY RESEARCH ACCOMPLISHMENTS.

- Generation of stable T cell lines cells that e xpress a m utated elongation factor to confer resistance to PEA toxin and that expre ss the IT gene regulated by CMV or CD40L promoter.
- Characterization of the immunotoxin-modified T cell lines.
- Evidence that the phenotype and function of the imm unotoxin–modified T cells are not altered by the IT genetic manipulation.
- Evidence that immunotoxin-modified T cells produce immunotoxins.

REPORTABLE OUTCOMES.
-Development of cell lines:
(CD40L Promoter) IT producing T cell lines
(CMV Promoter) IT producing T cell lines

CONCLUSIONS.
We have shown that the IT cloned in our lentiv iral vector can be produc ed by transduced cells using western-blot an d immunoprecipitation. T he m utated el ongation f actor can protect such cells from t he toxin they produced. W e have now protected our T cell lines with the sam e mutated elongation factor. Our recent data shows that T c ells c an be ef ficiently modif ied with our lentiviral vector encoding the immunotoxin w ithout impairing their phenotype and function. Indeed, T cells m odified with the immunotoxin surviv e, proliferate, m igrate and kill target cells.

Furthermore modification of these T cells did not induce their transformation (no tumors were generated by the genetic modification). These data revealed that the transduction with the elongation factor also protected T cells and that the production of the immunotoxin was not detrimental to their native phenotype and function. Our results also showed that the immunotoxin potentiate the antitumor efficacy of modified T cells.

We will continue to test the function and phenotypes of IT producing T cell and CTL lines from various patients and healthy donors. We will use 5 donors and 5 patient's cell lines (confirm the feasibility of the approach).